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Pre-emptive intrathecal Mk-801, a non-competitive N-methyl-D-aspartate receptor antagonist, inhibits the up-regulation of spinal dynorphin mRNA and hyperalgesia in a rat model of chronic inflammation

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Abstract

The effects of *N*-methyl-D-aspartate(NMDA) receptor antagonist, Mk-801, on the expression of spinal dynorphin (DYN) mRNA and the hyperalgesia induced by peripheral inflammation were studied by Northern analysis and behavioral test. Following an unilateral injection of complete Freund's adjuvant (CFA) into the rat hindpaw, there appeared a significant hyperalgesia of inflamed hindpaw and up-regulation of ipsilateral spinal DYN mRNA; while the pre-emptive and continuous intrathecal administration of Mk-801 (10 μg/μl per h) could significantly suppress both the hyperalgesia and the up-regulation of spinal DYN mRNA induced by peripheral inflammation. The results suggest that NMDA receptor activation may contribute to the development and maintenance of the thermal hyperalgesia that is associated with the up-regulation of DYN expression in spinal dorsal horn.

Keywords: Mk-801; *N*-Methyl-D-aspartate receptor; Hyperalgesia; Dynorphin mRNA; Complete Freund's adjuvant; Spinal cord; Rats

The intraplantar injection of inflammatory agents (e.g. complete Freund's adjuvant (CFA)) induces both behavioral hyperalgesia and spinal neuronal hyperexcitability by an acute and unilateral inflammation at the injection site [9,10,17,20]. In recent years, considerable behavioral, electrophysiological, and anatomical evidences suggest that excessive activation of the *N*-methyl-D-aspartate (NMDA) receptor, a type of ionotropic glutamate receptor, plays a key role in the development of behavioral thermal and mechanical hyperalgesia and central hyperexcitability in rat model of inflammation [2,6,12,15–17].

More recently, our study has demonstrated that the lumbar dorsal horn neurons that can be activated by noxious stimulation exhibited both NMDAR1 expression and c-Fos-like immunoreactivities (unpublished data); while there is report that intrathecal (i.t.) administration of Mk-801 suppresses the expression of c-Fos expression in the spinal dorsal horn induced by peripheral noxious stimulation [3]. In an in vitro study, it has been demonstrated that activation of NMDA receptors plays a critical role in regulating the intracellular calcium concentration, and subsequently, the expression of immediate-early genes (including c-fos) in cultured neurons [1]. Because it has been generally accepted that Fos protein acts as a transcription factor that modulates the expression of other genes and that Fos protein always coexists with dynorphin (DYN) mRNA [14], while DYN mRNA is also significantly increased in the spinal dorsal horn following peripheral inflammation and hyperalgesia [10,20,21], it is interesting to examine if the activation of NMDA receptors is needed for the up-regulation of DYN

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mRNA expression at the spinal level during peripheral inflammation and hyperalgesia.

Male Sprague–Dawley rats, weighing between 200 and 250 g, were divided into four groups with nine rats per group: (1) rats with i.t. Mk-801 and unilateral CFA (Mk-801/CFA); (2) rats with CFA alone (normal/CFA); (3) rats with i.t. saline and CFA (saline/CFA); and (4) rats with Mk-801 alone (Mk-801/Naive). Under pentobarbital sodium (Nembutal, 45 mg/kg, i.p.) anesthesia, a 7.0–7.5 cm length of PE-10 tubing was inserted into the lumbar subarachnoid space. Three days later, mini-pumps (Alzet model 2001), delivering drug at a rate of 1 μl/h, were implanted subcutaneously on the rats' dorsal surface under the same anesthesia as above. Rats with signs of motor impairment were excluded. Mk-801 (RBI) was dissolved in normal saline and released at 10 μg/μl per h, while the vehicle rats received normal saline. The next day following implantation of minipumps, a volume of 0.2 ml of CFA (*Mycobacterium tuberculosis*; Sigma), suspended in an oil/saline (1:1) emulsion, was injected subcutaneously into the plantar surface of the left hindpaw. Paw withdrawal latency in response to a thermal stimuli [9] was tested 1 h before CFA injection and 2, 24, 48 and 72 h after CFA injection. After behavioral testing was completed at 72 h following CFA injection, when the peak increase of DYN mRNA had been achieved as predicted by previous study [10], the animals were deeply anesthetized with Nembutal, (60 mg/kg, i.p.) and exsanguinated by cardiac puncture. An 8 mm portion of the lumbar enlargement was removed and divided along the midline into ipsilateral and contralateral sides. In Mk-801/Naive rats, the spinal cord was not divided. The tissue was immediately frozen on dry ice and stored at minus70°C. Total RNA was purified with a cesium gradient [4], each sample containing three pieces of spinal cord. Equal amounts (10 μg) of denatured total RNA were separated on 1% agarose-formaldehyde gels and transferred to Nytran membrane by capillary transfer with 20 × SSPE. RNA was crosslinked to the membrane by baking at 80°C for 1 h. Probes were labeled with α-^{32}P dCTP by nick translation [18]. The DYN probe was a 1.7 kb *Eco*RI/*Pst*I rat preprodynorphin genomic clone fragment [5].

Membranes were hybridized to labeled probes (2×10^6 cpm/ml) overnight at 42°C in 10 ml hybridization buffer containing 5% dextran sulfate. Blots were washed by standard methods, the most stringent being 0.5× SSPE/0.1% sodium dodecyl sulfate at 60°C for 30 min. RNA loading of the northern blots was normalized by re-probing the membranes with a glyceraldehyde phosphate dehydrogenase (GAPDH) oligonucleotide (Oncogene Science), which was labeled with α-^{32}P dATP by a DNA-end labeling method [19]. GAPDH was chosen as a normalization control since its expression in nerve tissue was not regulated by the surgery or by agents used within the design of this experiment. Autoradiographs were produced by exposing the labeled membranes to Biomax film with intensifying screens at 70°C. The membranes were then exposed to a phosphor screen and analyzed using a phosphoimaging system for quantification. The RNA blot data were obtained from three separate RNA extractions.

Baseline of withdrawal latency to thermal stimuli, measured 1 h before CFA injection, exhibited no differences between rats with pre-emptive Mk-801 (10.2 ± 0.42 vs. 9.7 ± 0.50 s) and rats without Mk-801 (10.0 ± 0.48 vs. 10.3 ± 0.64). After CFA injection, the withdrawal latency of the hindpaw contralateral to the CFA-injection remained unchanged, suggesting that Mk-801 had little effects on non-inflamed hindpaw. However, as shown in Fig. 1, there was a significant reduction of withdrawal latency, characterized as thermal hyperalgesia, of the hindpaw ipsilateral to the CFA-injection in both normal/CFA rats and Mk-801/CFA rats; the time course of the hyperalgesia was similar between these two groups of rats, with peak hyperalgesia at day 1 after CFA injection, but the hyperalgesia of the ipsilateral hindpaw of the Mk-801/CFA rats was significantly attenuated as compared to that of the normal/CFA rats ($P < 0.05$). The saline/CFA rats showed the same hyperalgesia as normal/CFA rats (data not shown). The withdrawal latency of rats with Mk-801 alone showed no difference in comparison to the baseline of other three groups of rats (data not shown), indicating that i.t. Mk-801 had little effects on hindpaw withdrawal in response to noxious stimuli in rats without CFA-injection.

Three days after CFA injection, there appeared an increased expression of DYN mRNA in the side of spinal cord ipsilateral to CFA injection in all CFA-injected rats. The normal/CFA and saline/CFA rats showed a 4-fold increase of DYN mRNA in the inflamed side as compared with that in the contralateral side. However, Mk-801/CFA

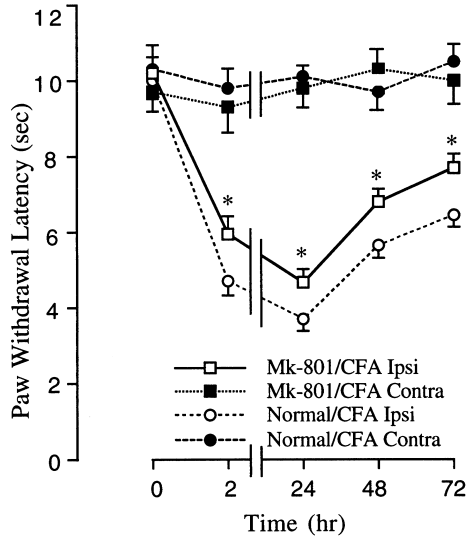

Fig. 1. Showing hindpaw withdrawal latencies (mean ± SEM) in response to noxious thermal stimulation in different experimental groups ($n = 9$ for each). Baseline withdrawal latencies were measured 1 h before CFA injections (time 0) and the latency changes were examined at different time following i.t. injection of normal saline or Mk-801. *$P < 0.05$ as compared to the withdrawal latency of ipsilateral hindpaw in rats of normal/CFA group.

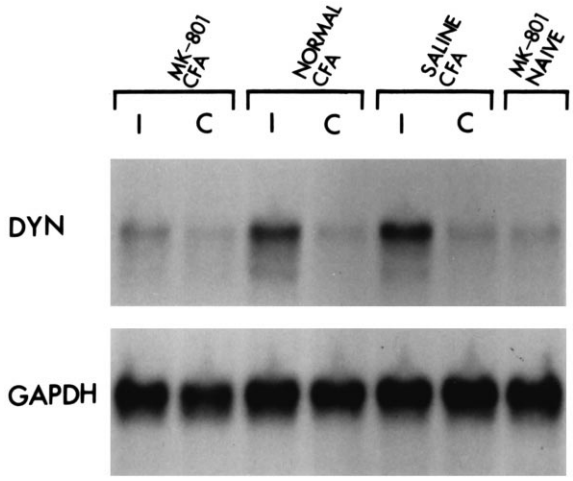

Fig. 2. Effects of NMDA receptor antagonist Mk-801 on the spinal DYN mRNA expression in lumbar spinal cord 3 days after unilateral CFA injection. Ten micrograms of total RNA was loaded in each lane with GAPDH expression as a loading control. The inflammation-induced up-regulation of DYN mRNA in ipsilateral side is dramatically lower in Mk-801/CFA rats as shown by comparison to that in same side of normal/CFA rats. The constitutive DYN mRNA expression in contralateral sides of CFA-injected rats and Mk-801/naive rats is pretty similar and lower. I, ipsilateral to the inflamed paw; C, contralateral to the inflamed paw.

rats exhibited only a 2-fold increase of DYN mRNA than that of contralateral side, though it is significantly lower ($P < 0.05$) than that in the inflamed side of normal/CFA and saline/CFA rats. The contralateral spinal cord of CFA-injected rats and the spinal cord of rats with i.t. Mk-801 alone rats showed very lower and pretty similar DYN mRNA expression. These data were summarized in Figs. 2 and 3.

The significant increases of DYN mRNA in spinal cord ipsilateral to CFA-induced peripheral inflammation and hyperalgesia in normal/CFA and saline/CFA rats are consistent with what has been reported previously [6,10,20]. The DYN mRNA expression in contralateral spinal cord of Mk-801/CFA rats exhibited as the same as that in the same side of normal/CFA and saline/CFA rats as well as of Mk-801/naive rats. This fact indicates that Mk-801 itself has no significant effects on the DYN mRNA expression in the spinal cord. This result is consistent with our behavioral study demonstrating no differences of withdrawal latency of contralateral hindpaw among CFA-injected groups and group with i.t. Mk-801 alone. Consistently, previous electrophysiological study has demonstrated that Mk-801 has no significant effects on receptive field size of dorsal horn neurons in rats without CFA-induced inflammation [17].

Of particular interest is that the spinal DYN mRNA expression in ipsilateral side in Mk-801/CFA rats is significantly lower ($P < 0.05$; Fig. 3) than that in saline/CFA and normal/CFA rats. It is obvious that pre-emptive administration of Mk-801 prior to the CFA injection and its continuous application during the development of inflammation significantly inhibits the up-regulation of spinal DYN mRNA expression induced by peripheral inflammation. This implies that the increased release of glutamate in spinal cord during peripheral inflammation [21] may induce the up-regulation of spinal DYN mRNA via operating on the NMDA receptors. For it has also been demonstrated that c-Fos protein, induced by peripheral inflammation, coexists with DYN mRNA [14] and that antisense c-fos mRNA can inhibit the induction of both c-Fos protein and DYN mRNA by formalin injection [7], it could be deduced that Mk-801 may inhibit the up-regulation of spinal DYN mRNA by way of suppressing the expression of c-Fos protein.

It should be pointed out that dynorphin has been considered to be biphasic in its modulatory effects on neuronal activity [11], though the present experiments persuade us to emphasize the nociceptive other than the antinociceptive effect of dynorphin. It has been postulated by Dubner and Ruda [6] that dynorphin-containing local circuit neurons are excitatory. Thus, the up-regulation of DYN mRNA would enhance the activity of dynorphin-containing spinal neurons and facilitate transmission in pain pathways. This proposal is supported by the previous investigations: direct application of dynorphin to the surface of the spinal cord produces expansion of the receptive fields in some dorsal horn nociceptive neurons [8]; intrathecal kappa opioid agonists produce hyperalgesia in the guinea pig [13]; and both DYN mRNA up-regulation and hyperalgesia induced by periph-

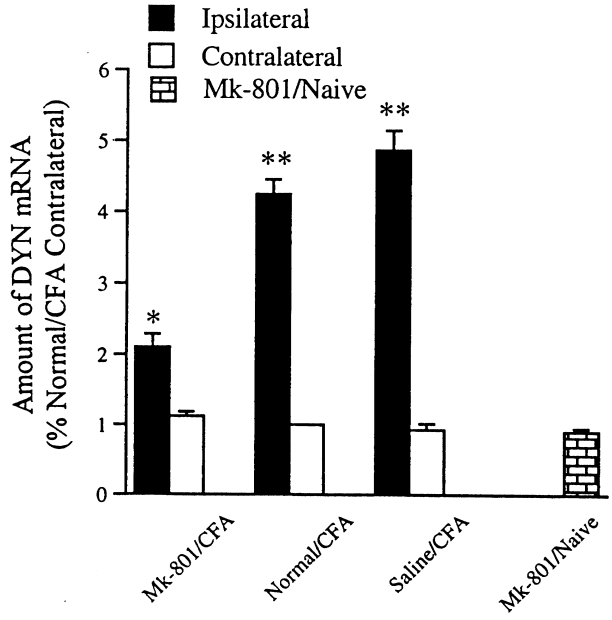

Fig. 3. Quantification of spinal DYN mRNA expression. Each bar represents the mean ± SEM of three different Northern blots made from three different RNA extractions, each extraction consisting of tissue from three rats in one experimental group. Blots were quantified on a phosphoimaging system, normalized to GAPDH; values were expressed as a percentage of the contralateral side of normal/CFA rats. The DYN mRNA up-regulation ipsilateral to the inflamed hindpaw in Mk-801/CFA, normal/CFA, and saline/CFA rats was 210, 421, and 470%, respectively. *$P < 0.05$ as compared to normal/CFA and saline/CFA rats; **$P < 0.01$ as compared to contralateral side.

eral inflammation are accompanied by an increase of DYN peptide in the spinal cord [10]. Therefore, it is reasonable to envisage that the attenuation of behavioral hyperalgesia in inflamed paw by Mk-801 in the present study is achieved, at least in part, by way of suppression of the up-regulation of DYN expression.

In conclusion, the present study suggests that NMDA receptors are involved in the up-regulation of DYN mRNA that leads to the development and maintenance of behavioral hyperalgesia and central hypersensitive state, though the involvement of other kinds of receptors in these processes could not be excluded completely.

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